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Change from Homo- to Heterolactic Fermentation by *Streptococcus lactis* Resulting from Glucose Limitation in Anaerobic Chemostat Cultures

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Lactic streptococci, classically regarded as homolactic fermenters of glucose and lactose, became heterolactic when grown with limiting carbohydrate concentrations in a chemostat. At high dilution rates (D) with excess glucose present, about 95% of the fermented sugar was converted to L-lactate. However, as D was lowered and glucose became limiting, five of the six strains tested changed to a heterolactic fermentation such that at $D = 0.1\ h^{-1}$ as little as 1% of the glucose was converted to L-lactate. The products formed after this phenotypic change in fermentation pattern were formate, acetate, and ethanol. The level of lactate dehydrogenase, which is dependent upon ketohexose diphosphate for activity, decreased as fermentation became heterolactic with *Streptococcus lactis* ML_3. Transfer of heterolactic cells from the chemostat to buffer containing glucose resulted in the nongrowing cells converting nearly 80% of the glucose to L-lactate, indicating that fine control of enzyme activity is an important factor in the fermentation change. These nongrowing cells metabolizing glucose had elevated (ca. twofold) intracellular fructose 1,6-diphosphate concentrations ($[FDP]_{in}$) compared with those in the glucose-limited heterolactic cells in the chemostat. $[FDP]_{in}$ was monitored during the change in fermentation pattern observed in the chemostat when glucose became limiting. Cells converting 95 and 1% of the glucose to L-lactate contained 25 and 10 mM $[FDP]_{in}$, respectively. It is suggested that factors involved in the change to heterolactic fermentation include both $[FDP]_{in}$ and the level of lactate dehydrogenase.

Group N streptococci (*Streptococcus cremoris*, *S. lactis*, and *S. diacetylactis*) play a vital role in many commercial milk fermentations, in which their primary function is to convert lactose to lactic acid (18). Lactic streptococci are useful because they possess limited metabolic diversity and usually convert about 95% of the fermented sugar to L-lactate (23, 26). This homolactic fermentation of either lactose or glucose occurs in batch culture when organisms are grown anaerobically near pH 7 at 30°C. In contrast, heterolactic fermentation was observed during growth on galactose (26) and during growth on lactose of variants defective in either lactate dehydrogenase (LDH; 21) or the lactose phosphotransferase system and/or phospho-β-D-galactosidase (7, 26), suggesting that these organisms have pathways which are not normally expressed. The alternative products reported include acetate, acetoin, CO_2, ethanol, formate, and glycerol. Unsuccessful attempts have been made with lactic streptococci to divert glucose fermentation products away from lactate by using procedures which cause other "homofermentative" lactic acid bacteria to become heterolactic. These procedures included growth without pH control (23) and aerobic growth on low concentrations of glucose (3).

Uptake of lactose and glucose by lactic streptococci involves phosphoenolpyruvate-dependent phosphotransferase systems, and glucose 6-phosphate is then metabolized to pyruvate via the glycolytic pathway. There is no evidence for other pathways of glucose metabolism having quantitative significance in lactic streptococci (for review, see 18). The galactose 6-phosphate moiety from the lactose molecule is metabolized via the D-tagatose 6-phosphate pathway (2) to triose phosphate intermediates. Pyruvate reduction to lactate in these streptococci involves an LDH whose activity in vitro is markedly dependent upon either fructose 1,6-diphosphate (FDP; 6, 17, 25) or tagatose 1,6-diphosphate (25). It has been suggested (7, 8, 26, 29) that the intracellular FDP concentration ($[FDP]_{in}$) may

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regulate LDH activity in vivo and, hence, the balance of fermentation products, but there is no experimental evidence for this with the lactic streptococci.

The purpose of this investigation was to examine regulation of end-product formation by using intact cells since, as Sanwal (24) has pointed out, proof is rare that mechanisms studied in vitro are actually the means of physiological regulation in vivo. Attempts were made to vary the intracellular concentration of LDH activator by using glucose-limited chemostat cultures and to examine the effect on end-product formation. Glucose was chosen as the growth sugar to simplify measurement of the intracellular concentration of LDH activator, and it was assumed that FDP was the only activator present.

MATERIALS AND METHODS

Organisms. *S. lactis* strains ML_3, ML_8, and H_1 and *S. cremoris* strains 158, 266, and AM_2 were from the collection held at the New Zealand Dairy Research Institute.

Growth media. *S. lactis* strains were grown in a chemically defined medium containing, per liter: (i) glucose, 5 g, unless otherwise specified; (ii) amino acids—cystine, glycine, histidine, isoleucine, leucine, methionine, phenylalanine, proline, threonine, tryptophan, tyrosine, and valine, 0.1 g each (L isomer); lysine and serine, 0.2 g each (L isomer); DL-alanine, L-asparagine, DL-aspartic acid, DL-glutamic acid, and L-glutamine, 0.3 g each; and L-arginine, 1g; (iii) bases—adenine, 5 mg; guanine, 1 mg; uracil, 5 mg; and xanthine, 5 mg; (iv) vitamins—*p*-aminobenzoic acid, 0.2 mg; biotin, 0.1 mg; folic acid, 0.1 mg; nicotinic acid, 1 mg; pantothenic acid, 1 mg; pyridoxal hydrochloride, 0.2 mg; pyridoxine hydrochloride, 1 mg; riboflavin, 0.1 mg; and thiamine hydrochloride, 0.1 mg; (v) other compounds—Tween 80, 0.2 g; disodium EDTA, 4 mg; $FeSO_4 \cdot 7H_2O$, 1 mg; $MnCl_2 \cdot 4H_2O$, 1 mg; $MgSO_4 \cdot 7H_2O$, 2.5 g; Na_2HPO_4, 2.5 g; and KH_2PO_4, 1 g. This medium was sterilized by passage through membrane filters (0.22-μm pore size; Millipore Corp.).

S. cremoris strains were grown in a complex medium containing, per liter; tryptone, 4 g; proteose peptone (no. 3), 2 g; beef extract, 2 g; yeast extract, 2 g (all products of Difco Laboratories); glucose, 5 g; $MgCl_2 \cdot 6H_2O$, 0.1 g; and K_2HPO_4, 3.5 g. This medium was autoclaved in two parts, and the glucose part was then added aseptically to the bulk medium.

Growth conditions. Cultures were grown in Porton-type chemostats (16) of 500-ml working capacity. The temperature was maintained at 30°C, and the pH was controlled at 6.5 ± 0.1 by automatic addition of NaOH. Anaerobic conditions were maintained by passing a 95% N_2–5% CO_2 mixture through the stirred culture (9). Medium was pumped into the fermentor at the appropriate dilution rate (*D*, proportion of culture volume replaced per hour), and the culture was allowed to reach equilibrium (i.e., grown for at least 10 mean generation times at each *D*) before being sampled.

Culture purity was checked daily by microscopic examination and by streaking on nutrient agar plates which were incubated at 30 and 37°C both aerobically and anaerobically in an N_2-CO_2 (95:5) atmosphere. In addition, cultures were tested periodically for lysis by their homologous phages and for agglutination with group N antisera (Wellcome Research Laboratories, Beckenham, England).

Sampling procedures. (i) Residual glucose and metabolic products. Because of the high glycolytic rate of growing lactic streptococci (ca. 0.4 μmol of glucose utilized per mg [dry weight] of bacteria per min), it is important that cells are rapidly removed from chemostat culture samples required for residual glucose and end-product analyses. Therefore, culture samples (ca. 10 ml) were cooled rapidly to about 10°C and passed through Millipore membranes (0.8-μm pore size) such that the total time between the sample leaving the chemostat and the end of filtration was <15 s.

(ii) Preparation of cell extracts. From the glycolytic rate it can be calculated that, at an $[FDP]_{in}$ of 25 to 30 mM, the cellular content of FDP could be metabolized by cells, held in the absence of exogenous glucose, within a few seconds. Therefore, an extremely rapid sampling and extraction procedure was required, which avoided the delays involved in methods using cell separation by filtration or centrifugation (22). Perchloric acid (0.3 ml, 9.2 N) was placed in a plastic syringe (5-ml capacity), the barrel was inserted, and air was displaced from the syringe. A sterile needle (11 cm long, 1.65-mm ID; with a tap) was fitted, the tap was closed, and the syringe barrel was drawn back and fixed at the 5-ml mark, leaving a vacuum in the syringe. The needle was inserted through a septum into the chemostat culture. When the tap was opened, culture was sucked into the syringe where turbulent mixing with the $HClO_4$ occurred. The average time between cells leaving the fermentor and mixing with $HClO_4$ was ca. 30 ms, and the final $HClO_4$ concentration was 0.6 N. Samples were held for 2 min at room temperature and then in an ice bath for 10 min before addition of 0.23 g of K_2CO_3. Neutralized (pH 7.0 to 7.5) samples were centrifuged and supernatants were stored at −90°C until assayed. With these procedures, glycolytic intermediates were stable and extraction was maximized.

To examine the effect of sampling time on $[FDP]_{in}$ (see Table 4), 10-ml culture samples were membrane filtered (0.8-μm pore size, 47-mm diameter; Millipore Corp.), and the filters plus retained cells were placed in 2 ml of 0.6 N $HClO_4$ at 0°C. The time taken for these manipulations was progressively increased so that the interval between cells leaving the fermentor and suspension in $HClO_4$ varied from 20 to 60 s.

Assay of residual glucose and metabolic products. (i) Glucose. Glucose ws determined with Glucostat reagents (Worthington Biochemicals).

(ii) Lactate. L-Lactate was measured enzymatically (Lactate UV Test, Boehringer Mannheim Corp.), and total lactate was determined colorimetrically (1).

(iii) Volatile products. Culture filtrates were analyzed for volatile products with a gas chromatograph (Pye Unicam series 104) and glass columns (1.5 m by 4 mm ID) packed with (a) 10% polyethylene glycol

adipate on Phasesep CL Aw 80–100 mesh and (b) Poropak Q. After sample injection, column (a) was held at 100°C for 4 min and then programmed at 3°C/min to 200°C and held for 15 min. Column (b) was held at 150°C for 6 min and then programmed at 12°C/min to 195°C. The carrier gas was O_2-free N_2 flowing at 40 ml/min. Detection was by flame ionization for column (a) and by Katharometer for column (b). Quantitation was carried out by a reporting integrator (Hewlett-Packard HP 3380A) programmed beforehand with authentic standards. Samples (0.25 ml) for acetate and ethanol analysis were acidified (6 N H_2SO_4, 20 μl) just before injection into column (a). For formate analysis, filtrate samples (1 ml) were made alkaline (ca. pH 8.0) with NaOH, dried, and dissolved in 0.5 N H_2SO_4 (0.1 ml), and samples (1 to 5 μl) were injected into column (b).

(iv) Nonvolatile acids. Culture filtrates (0.5 ml) were dried in vacuo before heating in 2 N methanolic hydrochloric acid (1 ml) in a sealed tube at 65°C for 2 h. The methyl esters were removed by ether extraction (three 3-ml extractions) and dissolved in dichloromethane after removal of the ether in a stream of N_2. Portions (1 to 5 μl) were then applied to column (a).

Uninoculated media were included as blanks for all end-product analyses, and the volume increase due to the addition of alkali for pH control in chemostat cultures was corrected for. The detection limits for glucose, formate, acetate, and ethanol were 5, 75, 50, and 50 μg/ml, respectively. In a search for other end products, column temperatures were varied from those given above.

Assay of glycolytic intermediates in cell extracts. The need for extremely rapid sampling and extraction of cells, without separation from the growth medium, has three important consequences for the assay of glycolytic intermediates: (i) the concentration of these compounds in extracts is low, such that assay requires enzymatic analysis by fluorimetry; (ii) culture filtrates must be assayed to check for release of intermediates from cells, and the appropriate subtractions from culture extract samples must be made; (iii) any growth medium constituents which fluoresce under the assay conditions will give high backgrounds. Although the defined medium used in this study was satisfactory in this regard, the relatively high fluorescence of the complex broth precluded assay of FDP in extracts from cultures growing in this medium.

Intermediates were assayed with a Perkin-Elmer fluorescence spectrophotometer (model MPF-2A) and NADH-coupled indicator systems as previously described (5, 28). Corrections were made for the difference in fluorescence between NADH in solution and enzymatically bound NADH. Values for $[FDP]_{in}$ were calculated on the basis that 1 g (dry weight) of cells was equivalent to 1.67 ml of intracellular fluid (cytoplasm) (28).

LDH activity. Cells were disrupted by shaking for 2 min at 4°C with glass beads in a Mickle disintegrator. After centrifugation (35,000 × *g* for 10 min), NAD-dependent LDH activity was immediately assayed in supernatant samples, using saturating pyruvate (10 mM), FDP (0.2 mM), and NADH (0.3 mM) as described previously (25). Activity was proportional to enzyme concentration, and assays on duplicate cell extracts were in good agreement. Protein was determined by the biuret method (15).

Experiments with nongrowing cells. Culture samples (50 ml) were removed from the chemostat and centrifuged, and the cells were washed and resuspended in 10 ml of phosphate buffer (20 mM, pH 6.5) at 3 to 6 mg (dry-weight equivalent) of bacteria per ml. Portions (2 ml) of this suspension were placed in a Radiometer pH-stat apparatus (TTA 31 titration assembly linked to an ABU 12 Autoburette, pH meter 26, Titrator 11, and Titrigraph SBR 2c). The stirred suspension was adjusted to pH 6.50 and maintained at 30°C under an atmosphere of O_2-free N_2. Glucose (50 μl, 0.2 M) was added, and the rate of alkali (0.1 N NaOH) addition required for pH control at 6.50 was recorded until alkali addition ceased (6 to 12 min). The total volume of alkali added was measured, the suspension was then centrifuged, and the supernatant was assayed for L-lactate and residual glucose. For measurement of $[FDP]_{in}$ in cells metabolizing glucose, suspensions (5 ml) were similarly maintained and glucose (100 μl, 0.2 M) was added. When half of the glucose had been utilized, FDP was rapidly extracted from cells by the evacuated-syringe/$HClO_4$ procedure and assayed by methods described above.

Other procedures. Bacterial density was determined directly in all chemostat cultures by filtration of three 10-ml samples, using preweighed Millipore membranes (0.8-μm pore size, 47-mm diameter). After water washing, the membranes plus retained cells were dried to constant weight at 100°C; variation between triplicates was $<\pm 3\%$. Amino acids were assayed with a Technicon TSM amino acid analyzer. Cells were assayed for glucoamylase-specific glycogen by the method of Hamilton (11).

RESULTS

Growth characteristics of lactic streptococci in the chemostat. Batch growth of *S. lactis* ML_3 in the fermentor pot (30°C, pH 6.5) gave mean generation times of 52 and 35 min in chemically defined and complex broth media, respectively. In continuous culture, steady-state populations were maintained at dilution rates (D = 0.8 h^{-1} for defined medium; D at least 1.0 h^{-1} for complex medium) similar to the maximum values predicted from batch cultures. The limiting growth factor in chemostat cultures was usually glucose (or lactose). In some *S. lactis* cultures, arginine may also have become limiting (Fig. 1), although cell density was proportional to glucose concentration in glucose-limited cultures. Analyses indicated that the other amino acids were in excess at all D values when the medium contained 0.5% glucose. When excess glucose was present at D = 0.6 to 1.0 h^{-1}, the specific rate of glucose utilization (14) was 0.38 to 0.44 μmol/mg (dry weight) of bacteria per min with the six strains of lactic streptococci. No detectable glycogen was found in cells growing

at $D = 0.2$ to 0.8 h^{-1} in the presence of excess glucose.

Cell growth on the wall of the fermentor vessel was not apparent with any of the six strains used in this study. With one strain (*S. lactis* ML_3), however, a clumping phenomenon was observed in all growth media (i.e., complex or defined; sugar excess or limiting) which was a function of the growth rate. This strain grew as diplococci at $D > 0.75$ h^{-1} and $D < 0.35$ h^{-1}, but at intermediate values the cells aggregated to form large clumps containing several thousand cells.

Fermentation products in chemostat cultures with glucose or lactose limitation. Growth of *S. lactis* ML_3 in the presence of excess glucose gave homolactic fermentation, and at $D = 0.76$ and 0.64 h^{-1} about 190 mmol of lactate was formed per 100 mmol of glucose fermented (Table 1). At $D = 0.56$ h^{-1}, the residual glucose concentration in the culture was very low, and products other than lactate became detectable.

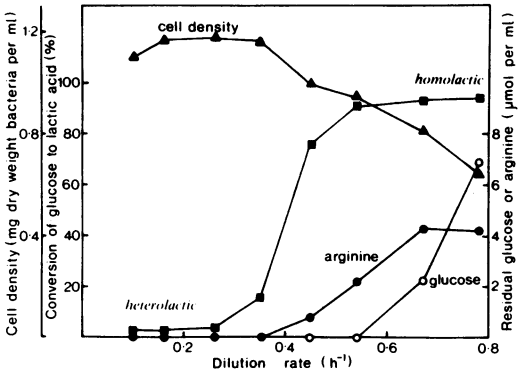

FIG. 1. *Cell density and fermentation pattern of S. lactis ML_3 growing in a chemostat in a chemically defined medium containing 25 mM glucose and 5.5 mM L-arginine.*

TABLE 1. *Residual glucose and fermentation products with S. lactis ML_3 growing in a chemostat*[a]

D (h^{-1})	Residual glucose (mM)	mmol of product/100 mmol of glucose fermented				Carbon recovery (%)
		L-Lactate	Formate	Acetate	Ethanol	
0.76	5.11	187	ND[b]	ND	ND	94
0.64	1.55	193	ND	ND	ND	97
0.56	0.03	185	16	13	9	103
0.48	ND	134	29	26	53	98
0.36	ND	38	134	73	88	95
0.25	ND	15	141	99	94	95
0.18	ND	7	125	111	103	96
0.11	ND	2	156	103	87	90

[a] Culture conditions: 30°C, pH 6.5, N_2-CO_2 (95:5) atmosphere, chemically defined medium containing 28 mM glucose.

[b] ND, Not detectable (see text).

As the dilution rate was progressively reduced in the absence of detectable glucose, the proportion of these products (formate, acetate, and ethanol) progressively increased until at $D = 0.11$ h^{-1} only 1% of the glucose was fermented to lactate. Data similar to those shown in Table 1 were obtained when the dilution rate was progressively increased from an initial low value.

The carbon recovery was close to 100%, allowing for the incorporation of glucose carbon into cellular material (normally about 3% with these organisms [13]). Gas-liquid chromatography revealed no trace of other products such as acetaldehyde, acetoin, acetolactate, butylene glycol, diacetyl, fumarate, pyruvate, and succinate. Enzymatic assays for L-lactate were in good agreement with the chemical assay for total lactate, indicating that no D-lactate was formed.

When the glucose in the growth medium was replaced by lactose (14 mM), a diversion of end products similar to that found with glucose (Table 1) was observed when lactose became limiting (data not shown).

Fermentation products in chemostat cultures with excess glucose. To determine whether the change in fermentation products was a function of growth rate or sugar limitation, the glucose concentration in the defined medium was increased to 0.22 M so that an excess (>25 mM) was present in the culture at all dilution rates. L-Lactate production was only slightly suppressed with *S. lactis* ML_3 as the dilution rate was decreased. At $D = 0.2$ and 0.1 h^{-1}, for example, 89 and 83%, respectively, of the glucose fermented were converted to L-lactate (Table 2). The limiting nutrient in these cultures was arginine.

Molar growth yields. The only known energy sources for *S. lactis* are carbohydrates and arginine (which is converted to ornithine with the production of 1 mol of ATP per mol of arginine utilized). The cell density of *S. lactis* ML_3 growing in chemostat cultures reflects not only the residual glucose and arginine concentrations, but also the change from homo- to heterolactic fermentation (Fig. 1). At $D = 0.78$ and 0.67 h^{-1}, when glucose was still in excess and fermentation was homolactic, the molar growth yields ($Y_{glucose}$) calculated from the data plotted in Fig. 1 were, respectively, 35.9 and 36.1 g (dry weight) of bacteria per mol of glucose fermented. When glucose became limiting, arginine was catabolized (Fig. 1), and a corresponding amount of ornithine appeared in the culture (data not shown). At $D = 0.35$ to 0.16 h^{-1}, when fermentation was heterolactic, the apparent $Y_{glucose}$ value was 46.8 g/mol. Subtraction of the theoretical contribution from arginine catabolism gave a corrected $Y_{glucose}$ of 43.8 g/mol. Therefore,

TABLE 2. *Regulation of end-product formation in S. lactis ML₃*[a]

Expt	Chemostat				Nongrowing cells[b]	
	D (h^{-1})	Conversion of glucose to L-lactate (%)	$[FDP]_{in}$[c] (mM)	LDH[d] sp act	Conversion of glucose to L-lactate (%)	$[FDP]_{in}$
i	0.76	94	25.2	12.4	99	26.8
	0.64	97	25.5	14.1	103	25.2
	0.56	93	21.2	18.1	99	27.4
	0.48	67	17.6	19.6	100	ND
	0.36	19	15.0	8.9	81	ND
	0.25	8	14.1	4.9	77	24.2
	0.18	4	11.0	4.1	78	28.7
	0.11	1	10.3	4.6	83	25.9
ii	0.20	89	27.7	10.4	99	ND
	0.10	83	19.8	9.2		ND

[a] Experiment i was described in Table 1; the medium contained 28 mM glucose, and chemostat cultures became glucose limited at D values below 0.56 h^{-1} (percent conversion of glucose to lactate derived from data in Table 1). In experiment ii, the medium contained 0.22 M glucose so that glucose was always in excess (>25 mM).

[b] Cells were removed from the chemostat and placed in buffer (see text). ND, Not determined.

[c] Intracellular FDP concentration (30-ms sampling time).

[d] LDH activity (micromoles of NADH oxidized per milligram of protein per minute).

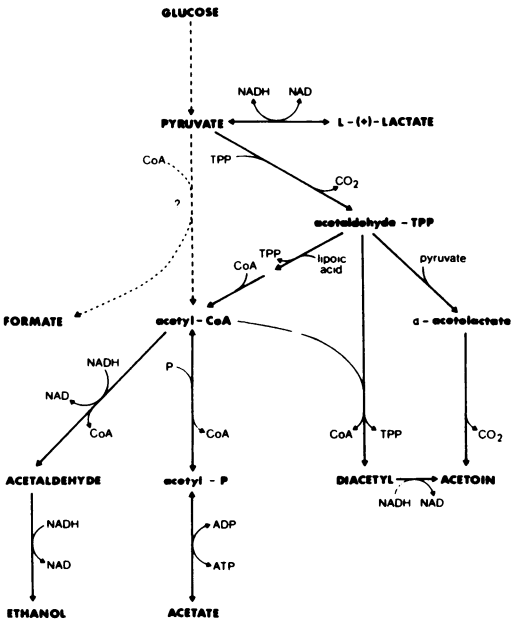

FIG. 2. *Alternative pathways for pyruvate metabolism in homofermentative lactic streptococci. CoA, Coenzyme A.*

associated with the change to heterolactic fermentation was a $Y_{glucose}$ increase from ca. 36 to 44 g/mol, which is consistent with the amount of acetate produced (Table 1) by the pathway shown in Fig. 2. These $Y_{glucose}$ values were not corrected for maintenance energy requirements which appear to be low with these organisms. For *S. lactis* ML_8, which remained homolactic with limiting glucose (Table 3), $Y_{glucose}$ did not increase at low D values.

Distribution of heterolactic potential among the lactic streptococci. Two additional strains of *S. lactis* (H_1 and ML_8) and three strains of *S. cremoris* (AM_2, 158, and 266) were examined in experiments similar to that described for *S. lactis* ML_3 in Table 1. Fermentation with all *S. cremoris* strains and with *S. lactis* H_1 became heterolactic with the production of formate, acetate, and ethanol (data not shown). The changes with *S. lactis* H_1 were similar to those observed with *S. lactis* ML_3, and, in the first steady state in which glucose was limiting ($D = 0.51\ h^{-1}$) formate, acetate, and ethanol were produced. Diversion to these products increased as D was lowered until at $D = 0.1\ h^{-1}$ about 7% of the glucose was fermented to L-lactate. All *S. cremoris* strains remained homolactic in the first two steady states in which glucose was limiting ($D = 0.6$ and $0.4\ h^{-1}$), but at lower D values lactate formation was suppressed until at $D = 0.1\ h^{-1}$ less than 20% of the glucose was fermented to L-lactate.

In striking contrast to the above-mentioned five strains, *S. lactis* ML_8 remained essentially homolactic when cultures were glucose limited at low D values (Table 3). Small quantities of acetate were produced, but no formate or ethanol was detected. With this strain the recovery of glucose carbon was consistently near 85% in separate experiments. Gas-liquid chromatography revealed no trace of the missing carbon.

TABLE 3. *Fermentation characteristics of S. lactis ML_8 growing in a chemostat*[a]

D (h^{-1})	Residual glucose (mM)	mmol of product/100 mmol of glucose fermented[b]		$[FDP]_{in}$[c] (mM)	LDH sp act[d]
		L-Lactate	Acetate		
0.61	3.42	171	ND	28.7	15.1
0.45	1.59	167	1	24.3	16.9
0.29	ND	166	6	28.9	11.6
0.15	ND	178	6	25.7	10.9
0.06	ND	164	10	24.2	8.8

[a] Culture conditions as for Table 1.

[b] Formate and ethanol were not detectable (ND).

[c] Intracellular FDP concentration (30-ms sampling time).

[d] Micromoles of NADH oxidized per milligram of protein per minute.

Does the switch to heterolactic fermentation involve phenotypic or genotypic change? A chemostat can provide a strongly competitive environment for the selective growth of different genotypes (see 10). Therefore, to distinguish between these two possibilities, the kinetics of the transition process from hetero- to homolactic fermentation were examined with *S. lactis* ML_3 (Fig. 3). Conditions in a heterolactic chemostat culture were changed by increasing D from 0.1 to 0.66 h^{-1}, at which point it was anticipated that fermentation would eventually become homolactic. At intervals after the transition, culture samples were assayed for glucose, lactate, formate, acetate, and ethanol. Excess glucose was present in the culture within 0.5 h of the transition. The rate of washout of products other than lactate approximated the theoretical washout rate (Fig. 3). This indicates that a phenotypic change occurred upon adjustment of the growth conditions such that the heterolactic cells immediately changed to homolactic fermentation. This transition would have been much slower had cells with a heterolactic genotype been selected in the glucose-limited cultures (10).

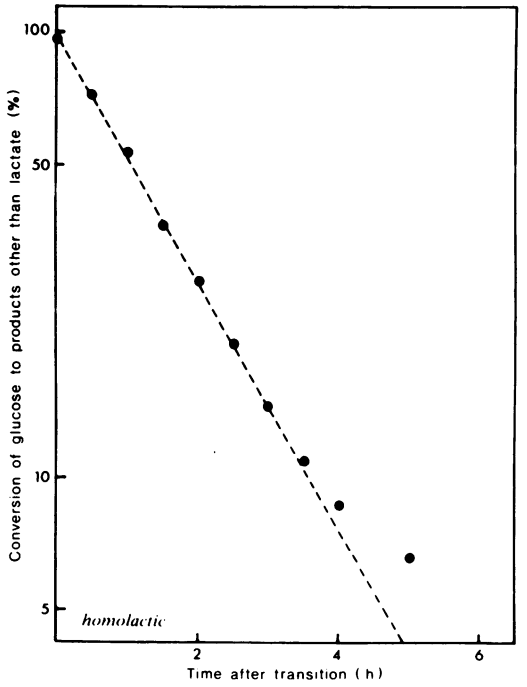

FIG. 3. *Kinetics of transition from hetero- to homolactic fermentation with S. lactis* ML_3. *A steady-state chemostat culture at* $D = 0.1\ h^{-1}$ *(glucose limited, converting only 2% of the available glucose to* L*-lactate) was changed at time zero to* $D = 0.66\ h^{-1}$: -----, *theoretical washout of products other than lactate at* $D = 0.66\ h^{-1}$, ●, *actual conversion of glucose to products other than lactate at* $D = 0.66\ h^{-1}$.

Regulation of end-product formation. (i) Glucose metabolism of cells after removal from chemostat. Growing *S. lactis* ML_3 cells were removed from chemostat cultures at each dilution rate and incubated anaerobically with glucose in a pH-stat apparatus (see Materials and Methods). To these nongrowing cells glucose was added, and the rate of alkali addition required for pH control at 6.50 was recorded. This rate, which corresponds to the rate of glucose utilization, was linear in all suspensions and varied between 0.21 and 0.15 μmol/mg (dry weight) of bacteria per min for cells from chemostat cultures growing at $D = 0.67$ and 0.10 h^{-1}, respectively (with nongrowing cells, the glucose utilization rate is about half that determined for cells growing in chemostat cultures with excess glucose). After the alkali demand had stopped, suspensions were assayed for L-lactate and residual glucose. Glucose was undetectable in all suspensions, and the conversion of glucose to lactate is given in Table 2. With cells derived from homolactic chemostat cultures, L-lactate accounted for ca. 100% of the glucose fermented in the nongrowing cell suspensions. Cells from heterolactic chemostat cultures (D = 0.36 to 0.11 h^{-1}) converted about 80% of the glucose to L-lactate suggesting that fine control of enzyme activity has an important influence on the balance of end products.

(ii) Intracellular concentration of FDP. With the rapid sampling and extraction procedure, the $[FDP]_{in}$ in *S. lactis* ML_3 cells growing with excess glucose was ca. 25 mM (Table 2). Although the $[FDP]_{in}$ declined as fermentation became heterolactic, the cells fermenting only 1% of the glucose to lactate still contained 10 mM FDP. Similar data were obtained in a separate experiment (Table 4, Fig. 1). Upon addition of excess glucose to a culture at $D = 0.2\ h^{-1}$ (Table 2) the $[FDP]_{in}$ increased to 28 mM, and fermentation became homolactic. With *S. lactis* ML_8, which did not deviate from L-lactate production, the $[FDP]_{in}$ remained high in glucose-limited cultures at low D values (Table 3). Sampling times of up to 60 s had no effect on $[FDP]_{in}$ values measured with glucose-excess *S. lactis* ML_3 cultures, but with glucose-limited cells the sampling time was obviously critical (Table 4). Nongrowing cells, derived from chemostat cultures at all D values, contained similar $[FDP]_{in}$ (ca. 26 mM) while fermenting glucose to L-lactate (Table 2).

Attempts were made to measure the intracellular concentrations of other glycolytic intermediates (glucose 6-phosphate, phosphoenol-

TABLE 4. *Effect of sampling time[a] on [FDP]$_{in}$ in S. lactis ML$_3$[b]*

D (h^{-1})	Residual glucose (mM)	[FDP]$_{in}$ (mM)			
		0.03 s[c]	20 s	40 s	60 s
0.78	6.85	26.5	27.9	26.4	25.1
0.67	2.23	27.1	24.5		
0.54	ND[d]	22.6	16.4		
0.45	ND	21.3	11.7		
0.35	ND	18.2	8.5		
0.26	ND	16.8	6.6		
0.16	ND	12.1	4.5		
0.10	ND	10.1	3.6	0.9	0.3

[a] Time between cells leaving chemostat culture and suspension in 0.6 N $HClO_4$.

[b] Other data from this experiment are shown in Fig. 1.

[c] Sampling time.

[d] ND, Not detectable.

pyruvate, and pyruvate) by using the rapid procedure which involved sampling without separation of cells from the medium followed by fluorometric enzymatic analysis. However, growing cells released small quantities of glucose 6-phosphate and pyruvate into the medium so that the concentration of these compounds in culture extracts could not be measured above the background level present in the cell-free culture samples. Phosphoenolpyruvate was obscured since the enzymatic assay procedure involved the intermediate formation of pyruvate. Although pyruvate was released from cells, the maximum concentration was too low for detection by gas-liquid chromatography.

(iii) LDH activity. The specific activity of LDH in *S. lactis* ML$_3$ increased when D was reduced from 0.76 to 0.48 h^{-1}, but further reduction in D resulted in a sharp decline in LDH activity (Table 2). A less-marked decline in activity was found with *S. lactis* ML$_8$ (Table 3). LDH specific activities were not affected by increasing the FDP concentration in the assay system from 0.2 to 2 mM. It seemed possible that LDH activity was inhibited by adenine nucleotides (4) and that the balance of these compounds changed in glucose-limited cells. However, AMP, ADP, and ATP had little effect on the in vitro activity of LDH from *S. lactis* ML$_3$ when tested at concentrations up to 10 mM in standard assay conditions with 0.05, 0.2, and 1 mM FDP.

DISCUSSION

L-Lactate was the only significant end product when lactic streptococci were grown with excess glucose in a chemostat at rates ranging from D = 0.8 to 0.1 h^{-1} (corresponding to mean generation times of 0.9 to 6.9 h). However, with a reduced glucose concentration in the growth medium, glucose became growth limiting, and end products were diverted from lactate to formate, acetate, and ethanol. Similar results were obtained with *Lactobacillus casei* (8) and *S. mutans* (9, 29). The likely pathways leading to the formation of these compounds from pyruvate in the lactic streptococci are shown in Fig. 2. The products suggest that, under conditions of limiting energy supply (glucose), the cells obtained ATP from pyruvate by a mechanism involving pyruvate formate-lyase. This is consistent with the $Y_{glucose}$ increase from 36 to 44 g/mol which accompanied the change to heterolactic fermentation. Glucose-limited *S. lactis* cells not only derived additional ATP from this change but also induced the enzymes for arginine catabolism (T. D. Thomas, unpublished data), which generates ATP. The product ratios expected from "phosphoroclastic" cleavage of pyruvate by pyruvate formate-lyase are formate-acetate plus ethanol (1:1). Since the observed ratios always showed a higher-than-expected proportion of acetate plus ethanol, it is possible that acetate was also produced from pyruvate via the intermediate formation of an acetaldehyde-TPP complex. The absence of detectable acetoin in heterolactic cultures is perhaps surprising in view of the pathways present in lactic streptococci (Fig. 2). Pyruvate formate-lyase has been found in *S. faecalis* (19) and *S. mutans* (30), but this highly O_2-sensitive enzyme has yet to be demonstrated in lactic streptococci. Of the six strains used in the present study, only one (*S. lactis* ML$_8$) remained homolactic under glucose limitation, although small amounts of acetate were produced, suggesting the absence of pyruvate formate-lyase in this organism.

Regulation of pyruvate metabolism may involve fine control of enzyme activity by effectors and/or control of enzyme synthesis, although another possible explanation for the change to heterolactic fermentation upon glucose limitation is the selection of cells with a different genotype. Continuous cultures are known to provide a highly selective environment (10), and accumulation of lactose-negative mutants in chemostat cultures of lactic streptococci has been demonstrated (20). However, kinetic experiments (Fig. 3) indicated that, when heterolactic cells were supplied with excess glucose, homolactic fermentation was immediately resumed, indicating that the change was entirely phenotypic.

The specific activity of the FDP-activated LDH of *S. lactis* ML$_3$ increased as D was initially lowered, but the level then decreased, as lactate

production was suppressed, to about 25% of the maximum (Table 2). With *S. lactis* ML_8 the LDH level also fell (to a lesser extent), but this strain remained homolactic (Table 3). Transfer of heterolactic *S. lactis* ML_3 cells, having reduced LDH levels, from the chemostat to a buffer containing glucose resulted in 80% conversion of glucose to L-lactate. Although this indicated that fine control of enzyme activity was important, nongrowing cells containing maximal LDH specific activity converted nearly 100% of the glucose to L-lactate, suggesting that the level of LDH did have some effect. The specific activity of the other enzymes likely to be involved in pyruvate metabolism (Fig. 2) may also change in the different growth environments, but these were not determined.

Factors which may regulate the activity of enzymes involved in pyruvate metabolism include substrate concentrations, activator and inhibitor concentrations, and the internal pH. The only LDH activator likely to be present in cells growing on glucose is FDP. The change from heterolactic fermentation in the chemostat to 80% conversion of glucose to lactate by nongrowing *S. lactis* ML_3 cells was accompanied by a twofold increase in $[FDP]_{in}$ (Table 2). A decrease of similar magnitude accompanied the transition from homo- to heterolactic metabolism with cells growing in a chemostat, and $[FDP]_{in}$ fell to ca. 10 mM (Table 2). Crow and Pritchard (6) found that the FDP concentration required for half-maximal velocity of LDH from *S. lactis* C_{10} in vitro was 4.4 mM in the presence of P_i. This suggests that the $[FDP]_{in}$ in heterolactic cells would be sufficient to give LDH catalytic activity in vivo. The present results suggest that changes in $[FDP]_{in}$ alone are not sufficient to explain fully the diversion of end products. In addition, the $[FDP]_{in}$ for lactic streptococci growing in batch cultures on glucose and galactose were similar, and yet fermentation of these sugars was homo- and heterolactic, respectively (T. D. Thomas, unpublished data). It is concluded that the combined effect of changes in $[FDP]_{in}$ and LDH level may largely explain the changes in fermentation pattern observed in chemostat cultures. However, additional factors may also influence the in vivo activity of LDH and/or the other enzymes involved in pyruvate metabolism. The pool size of NADH and pyruvate may vary with growth conditions. P_i is a potent inhibitor of LDH from lactic streptococci (6, 17, 25) and increased the FDP concentration required for half-maximal velocity in vitro by 2,000-fold (6) so that changes in the intracellular level of P_i may affect LDH activity in vivo. Glucose limitation may induce pyruvate formate-lyase synthesis or could conceivably result in a shortage of ATP and lead to a fall in intracellular pH. It is interesting that *S. lactis* ML_8 maintained a high $[FDP]_{in}$ with limiting glucose (Table 3), and it is possible that this ability, together with the relatively smaller decrease in LDH specific activity, was responsible for the homolactic behavior of this strain under glucose limitation.

Yamada and Carlsson (29) grew *S. mutans* JC 2 in a chemostat at $D = 0.12\ h^{-1}$ under glucose and nitrogen limitation and observed homo- and heterolactic fermentation, respectively. Cells in both cultures had similar LDH levels, but whereas $[FDP]_{in}$ was high in homolactic cells, this LDH activator was apparently absent from heterolactic cells which contained high levels of phosphoenolpyruvate. It was concluded that changes in $[FDP]_{in}$ regulate LDH activity in vivo, and suppression of lactate production was attributed to an effect on this single enzyme (29). Later work, however, indicated that pyruvate formate-lyase was induced in glucose-limited cells and that this enzyme was inhibited by glyceraldehyde 3-phosphate (30). In these studies (29), cells were removed from the chemostat and centrifuged before FDP extraction. From the data for *S. lactis* (Table 4) it would be anticipated that these relatively slow procedures would allow extensive FDP metabolism in glucose-limited *S. mutans* during sampling. The need for rapid sampling and extraction of metabolites, which may otherwise be quickly depleted, has often been stressed (see 12 and 22). When nongrowing cells of *S. lactis* depleted exogenous glucose, intracellular FDP disappeared and there was a corresponding increase in the levels of phosphoglycerates and phosphoenolpyruvate (28).

Previous reports on lactic streptococci have suggested that fermentation of glucose and lactose under anaerobic conditions is invariably homolactic. Brown and Collins (3) attempted to divert end products by growing lactic streptococci in batch cultures with "low concentrations" (2 to 5 mM) of glucose, reasoning that the cells would have low $[FDP]_{in}$. However, fermentation remained homolactic, presumably because glucose uptake by these organisms involves a high-affinity phosphoenolpyruvate-dependent phosphotransferase system (K_m 15 μM; 27) so that glucose only became limiting near the point of exhaustion from the batch cultures. The present investigation demonstrates the unique potential of the chemostat, since it is doubtful whether this heterolactic metabolism could be expressed by lactic streptococci growing in batch cultures on glucose or lactose.

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